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# Regulation of Heme Oxygenase-1 Expression *In Vivo* and *In Vitro* in Hyperoxic Lung Injury

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Using hyperoxia as a model of oxidant-induced lung injury in the rat, we explored the regulation of heme oxygenase-1 (HO-1) expression *in vivo* and *in vitro*. We demonstrate marked increase of HO-1 messenger ribonucleic acid (mRNA) levels in rat lungs after hyperoxia. Increased HO-1 mRNA expression correlated with increased HO-1 protein and enzyme activity. Immunohistochemical studies of the rat lung after hyperoxia showed increased HO-1 expression in a variety of cell types, including the bronchoalveolar epithelium and interstitial and inflammatory cells. We then examined the regulation of HO-1 expression *in vitro* after hyperoxia and observed increased HO-1 gene expression in various cultured cells including epithelial cells, fibroblasts, macrophages, and smooth muscle cells. Increased HO-1 mRNA expression correlated with increased HO-1 protein *in vitro*, and resulted from increased gene transcription and not from increased mRNA stability. We show that transcriptional activation of the HO-1 gene by hyperoxia requires cooperation between the HO-1 promoter and an enhancer fragment located 4 kb upstream from its transcription site. Increased HO-1 gene transcription was associated with increased activator protein-1 (AP-1) binding activity and supershift of the AP-1 complex by antibodies to c-Fos and c-Jun after hyperoxia. Taken together, our data suggest that AP-1 activation may represent one mechanism mediating hyperoxia-induced HO-1 gene transcription. Lee, P. J., J. Alam, S. L. Sylvester, N. Inamdar, L. Otterbein, and A. M. K. Choi. 1996. Regulation of heme oxygenase-1 expression *in vivo* and *in vitro* in hyperoxic lung injury. *Am. J. Respir. Cell Mol. Biol.* 14:556-568.

Reactive oxygen species (ROS) have been increasingly implicated in the pathogenesis of a variety of diseases and important biologic processes, including carcinogenesis, atherosclerosis, aging, and inflammatory disorders (1, 2). Toxic effects of these oxidants, including the superoxide and hydroxyl radicals and hydrogen peroxide, commonly referred to as oxidative stress, can cause cellular damage by oxidizing nucleic acids, proteins, and membrane lipids (3, 4). The lung is one major target organ for injury by these ROS, generated

endogenously by inflammatory cells and by exogenous oxidants such as environmental pollutants, cigarette smoke, drugs, chemotherapeutic agents, and bacterial pathogens (5). One major lung disease in which the pathogenesis is largely oxidant-mediated is adult respiratory distress syndrome (ARDS). ARDS is a disease process characterized by noncardiogenic edema and influx of inflammatory cells into the lung, where the cells release toxic ROS capable of initiating or amplifying lung injury (6).

Hyperoxia when administered to rats produces pathophysiologic changes similar to those seen in ARDS (3-5, 7). This *in vivo* model is particularly relevant to clinical syndromes such as ARDS not only because it mimics human ARDS but also because hyperoxic treatment is often used as a therapeutic modality to support lung function in patients succumbing to respiratory failure (e.g., ARDS), which further increases the oxidant burden of the lung (8, 9). When adult rats are exposed to hyperoxia (> 95% O<sub>2</sub>), minimal lung edema or pleural effusion is observed during the first 48 h, but increases significantly between 48 and 60 h. These rats will uniformly die between 60 to 72 h of continuous hyperoxia (9). Increased gene expression of antioxidant enzymes (AOE), particularly manganese superoxide dismutase

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Abbreviations: reactive oxygen species, ROS; adult respiratory distress syndrome, ARDS; antioxidant enzymes, AOE; manganese superoxide dismutase, MnSOD; copper-zinc superoxide dismutase, CuZnSOD; heme oxygenase-1, HO-1; activator protein-1, AP-1; ribosomal RNA, rRNA; chloramphenicol acetyl transferase, CAT; oxygen, O<sub>2</sub>; antioxidant-response element, ARE.

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(MnSOD), copper-zinc superoxide dismutase (CuZnSOD), and extracellular superoxide dismutase has been shown to confer protection against hyperoxic stress. For instance, transgenic mice overexpressing MnSOD and CuZnSOD have increased resistance to toxic effects of hyperoxia, and mice lacking extracellular superoxide dismutase are more sensitive to hyperoxia (10-12). In view of this important functional role of these AOE against hyperoxic stress, intensive investigations have been performed to delineate the molecular regulation of AOE expression by the lung in response to hyperoxia. The regulation of AOE expression in the rat lung *in vivo* after hyperoxia is complex and exerted at different levels (9). For instance, current evidence suggests that increased steady-state messenger ribonucleic acid (mRNA) levels of MnSOD are observed by 48 h of continuous hyperoxia exposure, whereas mRNAs for CuZnSOD, catalase, and glutathione peroxidase do not change significantly (9, 13). Additionally, the enzyme activity levels of these AOE are variable, since hyperoxia increases catalase and glutathione peroxidase activities, decreases MnSOD enzyme activity but does not alter CuSOD enzyme activity (9).

We have recently shown that in addition to the alterations seen in AOE gene expression, other stress-responsive genes, including heme oxygenase-1 (HO-1), *c-fos*, *c-jun*, and CCAAT/enhancer-binding proteins (C/EBP)- $\beta$  and C/EBP- $\delta$ , were increased *in vivo* after hyperoxia (13). Our laboratory has since focused and extended its studies on the regulation and function of HO-1 in oxidant lung injury. Heme oxygenase (HO), a ubiquitous enzyme in higher eukaryotes, catalyzes the initial and rate-limiting step in the oxidative degradation of heme to bilirubin (14, 15). The enzyme, utilizing NADPH and molecular O<sub>2</sub>, cleaves a meso carbon of the heme molecule, producing biliverdin. Biliverdin is then converted to bilirubin by biliverdin reductase (16, 17). Two isoforms of HO, HO-1 (32 kD) and HO-2 (36 kD), have been shown to be the products of two distinct genes (18). Whereas HO-2 is a constitutive enzyme, HO-1 is highly inducible. Although heme is the major substrate of HO-1, a variety of nonheme products including heavy metals, cytokines, hormones, endotoxins, sulfhydryl reagents, and heat shock are also strong inducers of HO-1 expression (19-22). Much interest in HO-1 has been stimulated by recent observations that HO-1 is also highly induced in response to various agents causing oxidative stress. Oxidant stress produced by agents such as ultraviolet irradiation, sodium arsenite, and glutathione depletors is a potent inducer of HO-1 (23-26). Similar to protective effects of MnSOD against oxidant injury, recent studies suggest that HO-1 also plays important roles in cellular protection against oxidant injury (27-31). Studies have also shown that HO-1 may mediate protection against oxidant insults such as ultraviolet irradiation and heme *in vitro* (27-29). Nath and colleagues have provided *in vivo* data supporting the role of HO-1 in providing protection against oxidant-mediated rhabdomyolysis renal injury (30). Our laboratory has also recently observed that hemoglobin-induced HO-1 induction plays a key role in providing protection against oxidant-mediated endotoxic shock and lung injury (31).

In view of these important functional roles of HO-1 against oxidant injury, and the fact that very little, if any, information exists regarding the molecular regulation of HO-1

in response to oxidant stress such as hyperoxia, we have chosen to delineate the molecular regulation of HO-1 expression *in vivo* and *in vitro* after hyperoxia. We show high levels of HO-1 mRNA in the rat lung *in vivo* after hyperoxia. Increased HO-1 mRNA is accompanied by increased HO-1 protein and enzyme. Using cultured cells, we show that increased HO-1 gene expression after hyperoxia is dependent on gene transcription. Transcriptional activation of the HO-1 gene after hyperoxia depends on both the HO-1 gene promoter and a 5' distal enhancer fragment (SX2) located 4 kb upstream of the transcription site of the HO-1 gene. HO-1 gene transcription was also associated with activation of the activator protein-1 (AP-1) transcriptional complex, suggesting that one mechanism underlying hyperoxia-mediated induction of the HO-1 gene may be early activation of AP-1.

## Materials and Methods

### Animals

Pathogen-free Sprague Dawley rats (200 to 225 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and allowed to acclimate upon arrival for 7 days prior to experimentation. Animals were fed rodent chow and water *ad libitum*. Animals were exposed to hyperoxia (> 99% O<sub>2</sub>) at a flow rate of 12 liters/min in a 3.70-ft<sup>3</sup> plexiglass exposure chamber, and were supplied with rodent chow and water *ad libitum* during the exposures. At the start of the exposures the chamber was humidified for 10 to 15 min. At each time point during the exposure, animal(s) were removed from the chamber and immediately killed by decapitation. Lung tissues were removed and frozen in liquid nitrogen for subsequent RNA and protein extraction. These experiments were done according to the animal protocol approved by the Animal Care Use Committee of The Johns Hopkins University School of Medicine.

### Tissue Culture

Human lung fibroblasts (WI-38 and MRC-5), human lung epithelial cells (A549), murine alveolar macrophage cells (MHS), and murine peritoneal macrophage cells (RAW 264.7) were obtained from the American Tissue Type Collection (ATTC). Rat alveolar macrophages (NR 8383) were obtained from Dr. R. J. Helmke, University of Texas Health Science Center (San Antonio, TX). Primary cultures of rat aortic smooth muscle cells (passages 2 to 10) were generously provided by Dr. Michael Crow of the National Institute of Aging in Baltimore, MD. All cell types were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL Laboratories; Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and gentamicin (50  $\mu$ g/ml), except for A549 and NR 8383 cells. A549 and NR 8383 cells were maintained in Ham-F12 medium (Gibco BRL Laboratories). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. Cells were exposed to hyperoxia (95% O<sub>2</sub>/5% CO<sub>2</sub>), with the exception of experiments for Figure 6C, in a tightly sealed modular chamber (Billup-Rothberg, Del Mar, CA) at 37°C. Since hyperoxia inhibits cell growth, all experiments were conducted in quiescent confluent cells in serum free medium to avoid cell-density variability between control

and hyperoxia-exposed cells during the course of the experiment.

### Chemicals

Actinomycin D, cycloheximide, polymyxin B, and endotoxin (*Escherichia coli* serotype 055:B5) were purchased from Sigma (St. Louis, MO).

### RNA Extraction and Northern Blot Analysis

Total RNA was isolated by the STAT-60 RNAzol method, with direct lysis of cells or homogenization of lung tissues in RNAzol lysis buffer followed by chloroform extraction (Tel-Test "B" Inc., Friendswood, TX). Northern blot analyses were performed as previously described (22). Briefly, 10  $\mu$ g of total RNA was electrophoresed in a 1% agarose gel and then transferred to Gene Screen Plus nylon membrane (Dupont, Boston, MA) by capillary action. The nylon membranes were then prehybridized in hybridization buffer (1% bovine serum albumin [BSA], 7% sodium dodecyl sulfate [SDS], 0.5 M phosphate buffer, pH 7.0, 1.0 mM ethylenediamine tetraacetic acid [EDTA]) at 65°C for 2 h followed by hybridization in hybridization buffer containing  $^{32}$ P-labelled rat HO-1 complementary deoxyribonucleic acid (cDNA) at 65°C for 24 h. Nylon membranes were then washed twice in wash buffer A (0.5% BSA; 5% SDS; 40 mM phosphate buffer, pH 7.0; 1 mM EDTA) for 15 min each at 65°C, followed by washes in buffer B (1% SDS; 40 mM phosphate buffer, pH 7.0; 1.0 mM EDTA) for 15 min four times each at 65°C. Ethidium bromide staining of the gel was used to confirm the integrity of the RNA. To further control for variation in either the amount of RNA in different samples or loading errors, blots were hybridized with an oligonucleotide probe corresponding to the 18S rRNA after stripping of the HO-1 probe. Autoradiogram signals were quantified by densitometric scanning (Molecular Dynamics; Sunnyvale, CA). All densitometric values obtained for the HO-1 mRNA transcript (1.8 kb) were normalized to values for 18S rRNA obtained on the same blot. Quantitation of steady-state HO-1 mRNA level of treated cells is then expressed in densitometric absorbance units, normalized to control untreated samples and expressed as fold induction compared with controls.

### cDNA and Oligonucleotide Probes

A full-length rat HO-1 cDNA, generously provided by Dr. S. Shibahara of Tohoku University, Sendai, Japan (32), was subcloned into pBluescript vector. A *HindIII/EcoRI* digestion was performed to cut the 0.9 kb HO-1 cDNA insert out of the pBluescript vector. A 24-bp oligonucleotide (5'ACG GTA TCT GAT CGT CTT CGA ACC 3') complementary to the 18S rRNA was synthesized using a DNA synthesizer (Applied Biosystems; Foster City, CA). HO-1 cDNA was labeled with [ $\alpha$ - $^{32}$ P]dCTP using the random primer kit from Boehringer Mannheim (Boehringer Mannheim, Germany). 18S rRNA oligonucleotide was labelled with [ $\alpha$ - $^{32}$ P]ATP at the 3' end, using terminal deoxynucleotidyl transferase (TdT; Bethesda Research Laboratories; Gaithersburg, MD).

### Western Blot Analysis

Frozen tissues or cells were homogenized in lysis buffer containing NP-40 (10%). Protein concentrations of the lysates

were determined by Coomassie blue dye-binding assay (Bio-Rad Laboratories, Hercules, CA). An equal volume of double-strength SDS/sample buffer (0.125 M Tris-HCl, pH 7.4; 4% SDS; 20% glycerol) was added, and the samples were boiled for 5 min. Samples were subjected to electrophoresis in a 12% SDS-polyacrylamide gel (Novex; San Diego, CA) for 2 h at 20 mA. The gel was transferred electrophoretically (Bio-Rad Laboratories) onto a polyvinylidene fluoride membrane (Immobilon; Millipore, Bedford, MA) and incubated for 2 h in 5% nonfat powdered milk containing 1 $\times$  Tris-buffered saline and 1% polyoxyethylene sorbitan monolaurate (TTBS). The membranes were then incubated for 2 h with rabbit polyclonal antibody against rat HO-1 (1:1,000 dilution). Rat HO-1 antibody was purchased from Stress Gen (Vancouver, Canada). After three washes in TTBS for 5 min each, the membranes were incubated with goat anti-rabbit IgG antibody (Amersham, Arlington Heights, IL) for 2 h. The membranes were then washed three times in TTBS for 5 min each, followed by detection of signal by using ECL detection kit from Amersham.

### Immunohistochemistry

Methods previously described were used for immunohistochemistry studies (33). Briefly, formalin (10%)-fixed tissue sections were rehydrated with PBS and blocked with 2% nonfat dry milk prior to incubating with a 1:1,000 dilution of the primary antibody anti-rat HO-1 (Stress Gen) in 3% BSA overnight at 4°C. Sections were washed three times with PBS (5 min each). The secondary antibody, a biotinylated goat anti-rabbit IgG, was incubated with a dilution of 1:500 in 3% BSA at 37°C for 1 h and detected with peroxidase-conjugated avidin-biotin complex. Negative controls for the nonspecific binding included normal rabbit serum without primary antibody, or secondary antibody alone. The slides were lightly counterstained with hematoxylin. The slides were scanned with Kodak Slide Scanner RFS 2035 using the Photoshop software program of Macintosh, Inc. (Cupertino, CA), and color composite was printed using the Kodak Printer XL 770 Digital Continuous Tone. The Photoshop software program was used to assist the preparing of the composite image.

### Plasmids and Transfections

The construction and characterization of pMHOICAT, the mouse extended HO-1 promoter (1.3 kb) linked to the reporter gene chloramphenicol acetyl transferase (CAT), has been described previously (34). The construction of pMHOICAT $\Delta$ -33+SX2, which contains the 5' distal enhancer fragment of the HO-1 gene (SX2) linked to the minimal promoter and reporter gene CAT, has also been described previously (35) (Figure 8). The construction of pMHOICAT+SX2, the extended HO-1 promoter (1.3 kb) linked to the SX2 distal enhancer and reporter CAT gene, has also been described previously (34). The construction of pMHO3CAT, the 5' flanking region of the HO-1 gene up to but not containing the SX2 site (Figure 8) and linked to the reporter gene chloramphenicol acetyl transferase (CAT), has been described previously (34). The construction of pMHOICAT, which contains a large portion of the 5' flanking region of the HO-1 gene linked to the reporter gene CAT

(Figure 8), was done by cloning the 11.5-kb (-3.5 to -15 kb) *Bam* HI/*Bam* HI fragment of  $\lambda$ MOH2-1(36) into the *Bam* HI site upstream of the HO-1 promoter in plasmid pMHOICAT. Plasmids (10  $\mu$ g) were stably cotransfected into RAW 264.7 cells with pcDNA 3-Neo (1  $\mu$ g), a plasmid containing neomycin selection marker, using Lipofectin<sup>®</sup> Reagent (Gibco BRL Laboratories) according to manufacturer's protocol. The cells were transfected for 24 h, after which time the plates were washed twice with serum-free media and then incubated in DMEM containing 10% FBS, gentamicin 50  $\mu$ g/ml, and Geneticin<sup>®</sup> 100  $\mu$ g/ml (Gibco BRL Laboratories). At approximately 3 day intervals, the concentration of Geneticin<sup>®</sup> in the media was increased up to a maximum dose of 800  $\mu$ g/ml. The surviving colonies (neomycin resistant) on each plate were pooled to establish the sublines.

### CAT Assay

Cellular protein extracts were harvested within 24 h after termination of hyperoxia exposure. Cells were washed with cold PBS before resuspending cells in 1.0 ml of 0.125 M Tris-HCl (pH 7.5), and were then lysed by three cycles of freezing and thawing. Cell debris were then removed by centrifugation for 10 min at 14,000  $\times$  g. Protein concentrations of the supernatants used for CAT assays were determined by Coomassie blue dye-binding assay (Bio-Rad Laboratories). In a reaction mix of 150  $\mu$ l containing 20 mM acetyl CoA and 0.3  $\mu$ Ci [<sup>14</sup>C]chloramphenicol (Amersham), 100  $\mu$ g of protein were incubated for 16 h at 37°C. Equal amounts of protein were used for each sample. The amount of acetylation was then determined by counting the acetylated and nonacetylated forms, which were separated by ascending thin layer chromatography. Percentages of chloramphenicol acetylation were obtained over the linear range of the assay (30 to 50%) for each sample, and were normalized for the protein content of the extract. If the assay gave more than 50% conversion of chloramphenicol, the assay was repeated for a shorter period of incubation or with less cell extract. Mock transfections show a chloramphenicol conversion range of < 0.5%.

### Cellular Nuclear Protein Extraction

Cells were scraped in cold PBS and centrifuged at 14,000  $\times$  g at 4°C for 10 min. After the supernatant was discarded, the cell pellet was lysed in lysis buffer containing 10 mM HEPES, pH 7.9; 1 mM EDTA; 60 mM KCl; 1 mM dithiothreitol (DTT); 0.5% NP-40; and 1 mM PMSF. The lysate was chilled in ice for 5 min and then centrifuged at 1,500  $\times$  g to obtain cell nuclei. The nuclei were washed in lysis buffer without NP-40 and centrifuged again at 1,500  $\times$  g for 5 min. The supernatant was removed and the pellet was resuspended in nuclear resuspension buffer containing 25 mM Tris pH 7.8; 60 mM KCl; 1 mM DTT; and 1 mM PMSF. The nuclei were then frozen and thawed three times to obtain nuclear protein. The protein was kept in nuclear resuspension buffer and stored at -70°C. Protein concentrations used for electrophoretic mobility shift assay were determined by Coomassie blue dye-binding assay (Bio-Rad Laboratories).

### Electrophoretic Mobility Shift Assay

Mobility shift assays were performed as described by Barberis and colleagues (37), with minor modifications. Briefly, DNA-binding activity was determined after incubation of 1.0  $\mu$ g of RAW 264.7 nuclear protein extract with 10 femtomoles (20,000 to 50,000 cpm) of a <sup>32</sup>P-labeled 22-mer oligonucleotide encompassing the consensus AP-1 site (5'-CTAGTGATGAGTCAGCCGGATC 3') in reaction buffer containing 10 mM HEPES, (pH 7.9); 1 mM DTT; 1 mM EDTA; 80 mM KCl; 1  $\mu$ g poly [dIdC][dIdC] and 4% Ficoll. After a 20 min incubation, the reaction mixture was electrophoresed on a 6% polyacrylamide gel (2% bisacrylamide, 6.5% Tris borate-EDTA, 0.08% ammonium persulfate). The gel was transferred to DE81 ion-exchange chromatography paper (Whatman, Maidstone, England) and dried down prior to exposure to autoradiographic film. Self competitions were conducted under the same conditions, using 1-, 10-, and 100-fold (X) molar excess of the unlabeled AP-1 oligonucleotide probe. Nonspecific competitions were similarly performed using an unlabeled oligonucleotide probe encompassing an Sp1 transcription factor binding site (5'-GATCGATCGGGGCGGGGCGGATC 3') (Stratagene). c-Fos and c-Jun antibodies used for mobility gel supershift assays were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

### Heme Oxygenase Activity

Animals were anesthetized with sodium pentobarbital and exsanguinated via transection of the heart. Lungs were then perfused free of blood with sterile saline, excised, blotted, and homogenized in buffer containing 0.1 M Tris acetate, 0.1 M KCl, and 1 mM EDTA prior to microsomal HO activity determination (38). One unit of HO activity was defined as the amount of bilirubin (nmol) produced per milligram of protein per hour.

### Statistical Analysis

Data are expressed as the mean  $\pm$  SEM. Differences in measured variables between the experimental and control groups were assessed using Student's *t*-test. Statistical calculations were performed on a Macintosh personal computer using the Statview II Statistical Package (Abacus Concepts, Berkeley, CA). Statistical difference was accepted at *P* < 0.05.

## Results

### Induction of HO-1 Expression after Hyperoxia Exposure *in vivo*

Northern blot analyses were used to examine steady-state levels of HO-1 mRNA in rat lungs after hyperoxia exposure. Lungs were removed from rats at 0, 24, 48, and 64 h of hyperoxia exposure and analyzed for HO-1 mRNA expression. As shown in Figure 1 (*inset*), marked increase of steady-state HO-1 mRNA levels was observed at 48 and 64 h of hyperoxia exposure. 18S rRNA hybridization was used as a normalization control. Quantitation of HO-1 mRNA expression in the rat lungs shows a 1.9-fold induction by 24 h, 5.8-fold induction by 48 h, and 13.0-fold induction by 64 h of hyperoxia when compared with normoxic control rats

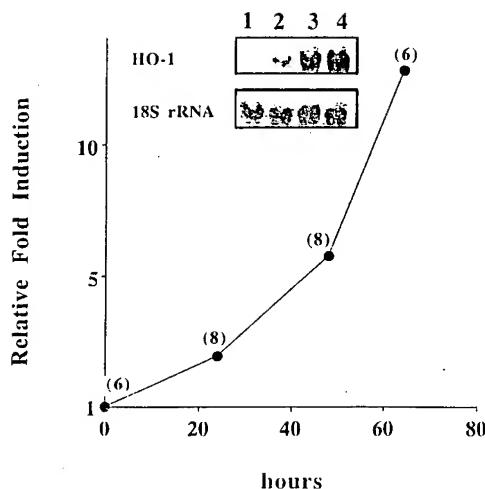


Figure 1. Kinetics of HO-1 mRNA expression in rat lungs after hyperoxia exposure. (Inset) Total RNA was extracted from lung tissues after hyperoxia and analyzed for HO-1 mRNA expression by Northern blot analysis. 1: Normoxia control; 2: 24 h hyperoxia; 3: 48 h hyperoxia; 4: 64 h hyperoxia. 18S rRNA hybridization is shown as a normalization control. (Graph) Quantitation of relative HO-1 mRNA levels in rat lungs after hyperoxia assessed by Northern blot hybridization. Results represent the mean fold induction of HO-1 mRNA levels in rats exposed to hyperoxia compared with normoxic rats from three independent experiments. ( $n$  = number of rats.)

(Figure 1, graph). To further examine whether the increased HO-1 mRNA levels were accompanied by increases in HO-1 protein levels, Western blot analyses were performed in rat lungs after hyperoxia. Figure 2 (inset) shows marked increases of HO-1 protein expression in the rat lungs at 48 and 64 h of hyperoxia. Quantitation of HO-1 protein expression showed a 2.0-fold increase by 24 h, 3.7-fold increase by 48 h, and 8.3-fold increase by 64 h of hyperoxia (Figure 2, graph). Increased HO-1 protein levels also correlated with increased HO-1 enzyme activity as shown in Figure 3. Increased HO-1 enzyme activity was observed in the rat lungs by 24 h, followed by a greater increase after 48 and 64 h of hyperoxia.

#### Immunohistochemical Analysis of HO-1 Expression in the Rat Lung after Hyperoxia Exposure

In order to further understand the regulation of HO-1 expression in the rat lung after hyperoxia *in vivo*, we attempted to localize the cell type(s) responsible for hyperoxia-induced HO-1 expression in the rat lungs. Immunohistochemical studies of lung tissues after hyperoxia exposure *in vivo* were performed using anti-rat HO-1 antibody. Figure 4A is a slide stained without primary antibody but with secondary antibody alone as a control for nonspecific staining. Figure 4B illustrates the scant basal levels of HO-1 expression in the

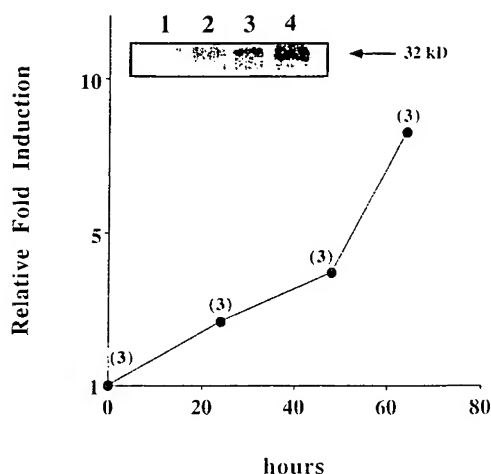


Figure 2. Kinetics of HO-1 protein expression in rat lungs after hyperoxia. (Inset) Total protein was isolated from tissues after hyperoxia exposure and subjected to Western blot analysis. 1: Normoxia control; 2: 24 h hyperoxia; 3: 48 h hyperoxia; 4: 64 h hyperoxia. (Graph) Quantitation of relative HO-1 protein levels in rat lungs after hyperoxia assessed by Western blot analysis. Results represent the mean fold induction of HO-1 protein levels in rats exposed to hyperoxia compared with normoxic rats from three independent experiments. ( $n$  = number of rats.)

lungs of untreated control rats. After hyperoxia exposure, diffuse increased staining for HO-1 protein was observed in the alveolar walls (Figure 4C). Figure 4D represents a higher magnification of the inset in Figure 4C, illustrating the prom-

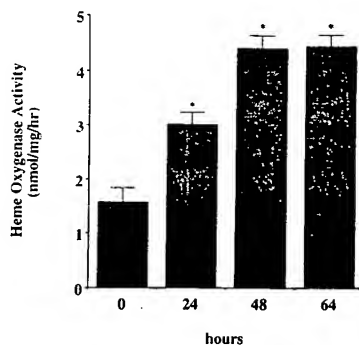
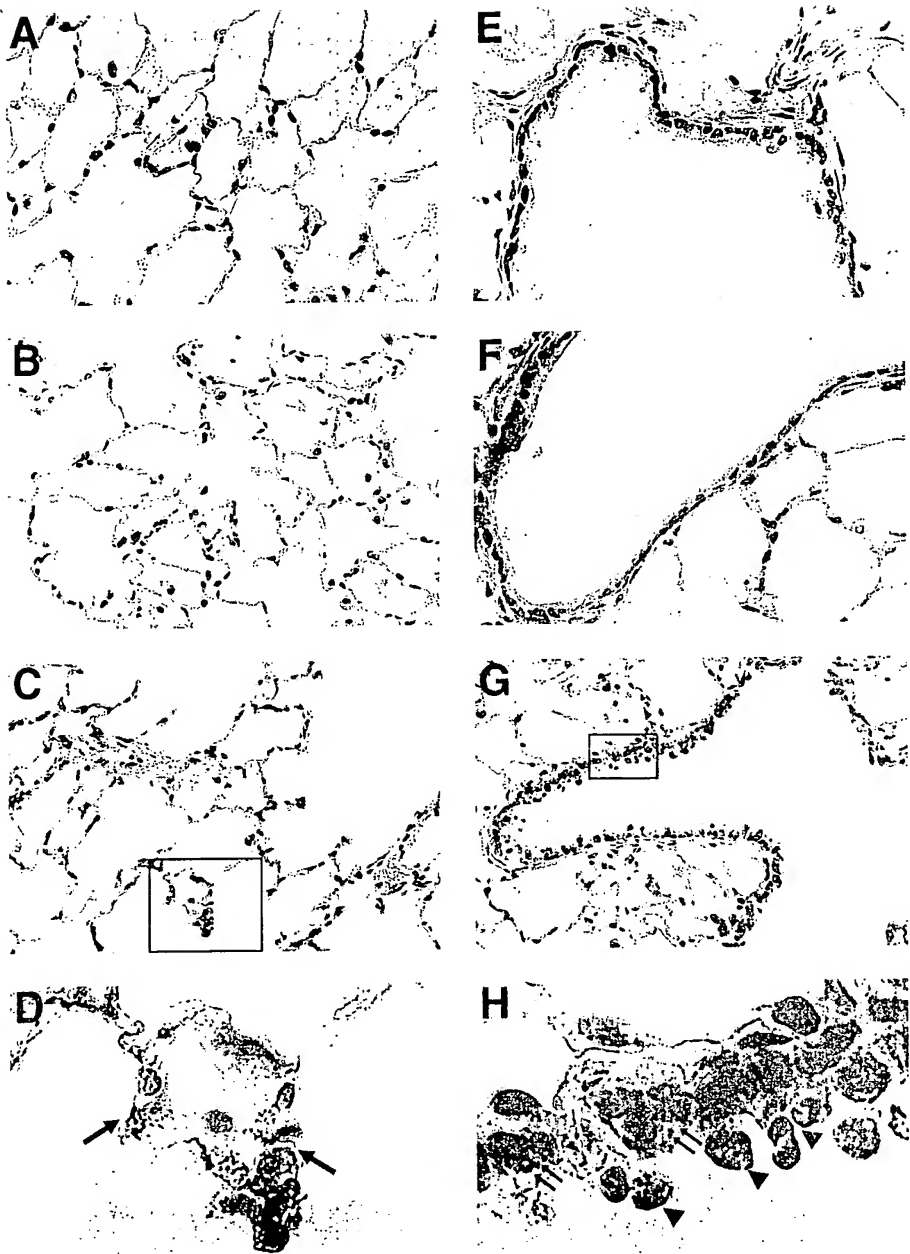


Figure 3. Kinetics of HO enzyme activity in rat lungs after hyperoxia exposure. HO enzyme activity in rat lungs after hyperoxia exposure determined by bilirubin generation. Enzyme activity represents mean  $\pm$  SEM of measurements from six rat lungs. \*  $P < 0.001$  compared with control values.



**Figure 4.** Immunohistochemical studies of rat lung after hyperoxia exposure. Formalin-fixed sections were processed for immunohistochemistry using anti-rat HO-1 antibody in lung tissue after 48 h of hyperoxia exposure. (A) A normoxia control slide incubated in the absence of primary antibody (original magnification:  $\times 26$ ). (B) Normoxia control rat (original magnification:  $\times 26$ ). (C) Hyperoxia 48 h (original magnification:  $\times 26$ ). (D) Hyperoxia 48 h (original magnification:  $\times 100$ ). (E) A normoxia control slide incubated in the absence of primary antibody (original magnification:  $\times 26$ ). (F) Normoxia control rat (original magnification:  $\times 26$ ). (G) Hyperoxia 48 h (original magnification:  $\times 26$ ). (H) Hyperoxia 48 h (original magnification:  $\times 100$ ). Single arrow = alveolar wall; double arrows = bronchiolar wall; arrowheads = inflammatory cells. All sections are counterstained with hematoxylin.

inent HO-1 protein staining in the alveolar wall (*single arrow*), predominantly in the alveolar epithelium of rats exposed to hyperoxia. Figure 4E through H illustrates the pattern of HO-1 expression in the bronchiolar wall. Figure 4E represents a slide stained without primary antibody, with secondary antibody alone, used as a control for nonspecific staining. As was the case for alveolar epithelium, untreated control rats revealed very low basal levels of HO-1 expression in the bronchiolar epithelium (Figure 4F). However, a marked increase of HO-1 protein expression was observed in the bronchiolar epithelium, with intense staining of HO-1 protein in the inflammatory cells after hyperoxia exposure (Figure 4G). Figure 4H represents a higher magnification of the inset shown in Figure 4G, showing the marked HO-1 staining in inflammatory cells (*arrowheads*) and bronchiolar epithelium (*double arrows*).

#### Increased HO-1 Expression in Systemic Organs after Hyperoxia Exposure

In order to determine whether induction of HO-1 after hyperoxia exposure is observed in other systemic organs, Northern blot analyses were performed on various organs including the liver, heart, spleen, kidney, aorta and adrenal gland after hyperoxia. Interestingly, these systemic rat tissues (except the aorta) all exhibited increased HO-1 mRNA expression after hyperoxia (Figure 5). Quantitative analyses showed highest levels of HO-1 mRNA in the kidney (49.7-fold induction), followed by the liver (21.3-fold induction), lung (7.2-fold induction), spleen (2.6-fold induction), adrenal glands (2.0-fold induction), and heart (1.3-fold induction).

#### HO-1 mRNA Induction Is Observed in Lung Cells *in vitro* after Hyperoxia Exposure

We next extended our studies to examine the regulation of HO-1 expression *in vitro* after hyperoxia exposure. We first tested several cell types, including RAW 264.7 macrophages, NR 8383 macrophages, MHS macrophages, WI-38 and MRC-5 lung fibroblasts, A549 pulmonary epithelial cells, and rat aortic smooth muscle cells for HO-1 mRNA expression after hyperoxia exposure *in vitro*. All cell types exhibited increased HO-1 mRNA expression after 24 to 48 h of hyperoxic exposure. Table 1 summarizes the relative level of HO-1 mRNA expression observed in these various cell types after 48 h of hyperoxia. Figure 6A shows representative Northern blot analyses of three different macrophage cell lines (RAW 264.7, NR 8383, and MHS) for HO-1 mRNA expression after hyperoxia exposure. The highest level of

TABLE 1  
Induction of HO-1 mRNA accumulation in various cultured cells by hyperoxia

Cell type	Relative HO-1 mRNA levels
MRC-5 lung fibroblasts	†
WI-38 lung fibroblasts	*
A549 lung epithelial cells	*
RAW 264.7 macrophages	‡
NR 8383 macrophages	†
MHS macrophages	*
Rat aortic smooth muscle cells	*

Total RNA was harvested 48 h after hyperoxia and analyzed for HO-1 mRNA expression by Northern blot analyses. Data are expressed as relative HO-1 mRNA levels compared to control untreated samples and are mean values from 3-4 independent experiments.

\* = 2- to 5-fold induction.

† = 6- to 10-fold induction.

‡ = > 10-fold induction.

HO-1 induction was observed in RAW 264.7 macrophages (13.0-fold), followed by mouse alveolar MHS macrophages (4.7-fold) and rat alveolar NR 8383 macrophages (3.5-fold).

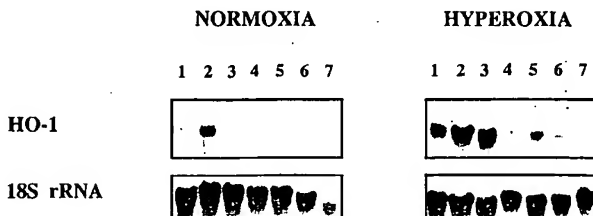
#### Kinetics and Dose-response of HO-1 mRNA Expression in RAW 264.7 Cells after Hyperoxia

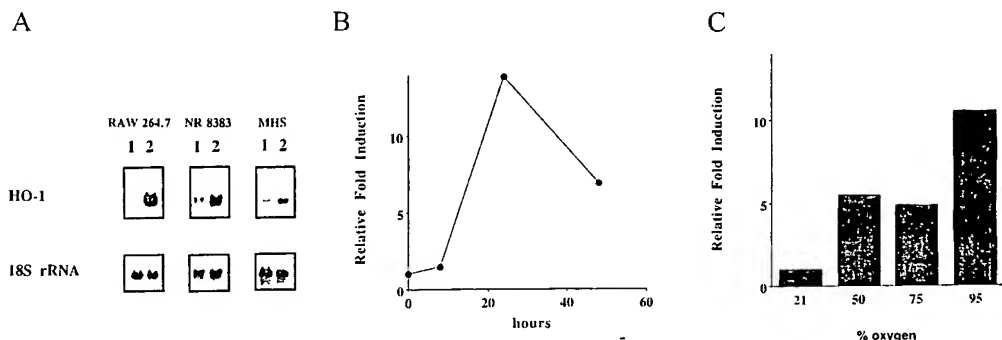
Figure 6B illustrates the kinetics of HO-1 mRNA induction in RAW 264.7 cells during continuous hyperoxia exposure, with peak induction at 24 h of hyperoxia exposure. HO-1 mRNA induction was dose dependent, since increasing levels of HO-1 mRNA were observed with increasing O<sub>2</sub> tensions. Compared with normoxic controls, cells exposed to 50% O<sub>2</sub> and 75% O<sub>2</sub> exhibited an approximately 5-fold increase of HO-1 mRNA levels (Figure 6C). Cells exposed to 95% O<sub>2</sub> showed a 10-fold induction of HO-1 mRNA compared with normoxic controls.

#### HO-1 mRNA Expression Correlates with Increased HO-1 Protein in RAW 264.7 Cells after Hyperoxia

To further examine whether increased HO-1 mRNA levels in RAW 264.7 cells were accompanied by increases in HO-1 protein levels, Western blot analyses were performed in cellular lysates from cells exposed to hyperoxic conditions. As with rat lungs exposed to hyperoxia *in vivo*, increased HO-1 protein was also observed in RAW 264.7 cells after hyperoxia *in vitro*, exhibiting increased protein levels at 8 and 24 h (Figure 7). LPS-treated cells were used as a positive

**Figure 5.** Northern blot analysis of HO-1 mRNA expression in various organs of rats after hyperoxia exposure. Total RNA was extracted from various organs after 48 h of hyperoxia and analyzed for HO-1 mRNA expression by Northern blot analysis. 1: Liver; 2: spleen; 3: kidney; 4: adrenal gland; 5: lung; 6: heart; 7: aorta. 18S rRNA hybridization is shown as a normalization control. Data shown are representative blot of two independent experiments.





**Figure 6.** Northern blot analysis of HO-1 mRNA expression in macrophages after hyperoxia exposure. (A) Total RNA was extracted from the indicated macrophage cell line after 24 h of hyperoxia and analyzed for HO-1 mRNA expression by Northern blot analysis. 1: Untreated control; 2: hyperoxia 24 h. 18S rRNA hybridization is shown as a normalization control. Data shown are representative blot of three independent experiments. (B) Kinetics of HO-1 mRNA expression in RAW 264.7 cells after hyperoxia exposure. Total RNA was isolated from cells at the indicated times after hyperoxia and analyzed for HO-1 mRNA expression by Northern blot hybridization. 18S rRNA hybridization was used as a normalization control. The results represent mean fold induction of HO-1 mRNA levels in hyperoxic cells compared with normoxic cells from five independent experiments. (C) Dose-response of HO-1 mRNA expression in RAW 264.7 cells after hyperoxia exposure. Total RNA was extracted from cells after 24 h of hyperoxia at the indicated  $O_2$  tension and analyzed for HO-1 mRNA expression by Northern blot analysis. 18S rRNA hybridization was used as a normalization control. The results represent mean fold induction of HO-1 mRNA levels in hyperoxic cells compared with normoxic cells from three independent experiments.

control for HO-1 immunoblotting, since we have previously observed increased HO-1 protein induction by endotoxin (22).

#### Transcriptional and Posttranscriptional Regulation of HO-1 mRNA Expression after Hyperoxia

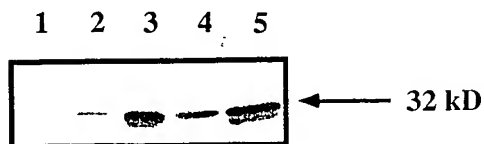
To further investigate the molecular basis of increased HO-1 gene expression, we examined whether HO-1 mRNA induction after hyperoxia is dependent on gene transcription. Table 2 summarizes experiments examining the effects of actinomycin D on hyperoxia-induced HO-1 gene expression. Pretreatment of cells for 1 h with actinomycin D, a potent inhibitor of RNA transcription, prior to hyperoxia exposure completely inhibited hyperoxia-mediated HO-1 mRNA induction (Table 2). To further confirm that the increased HO-1 gene expression after hyperoxic exposure is transcriptionally regulated, RAW 264.7 cells were first stably transfected with a plasmid containing the HO-1 gene linked to the CAT gene (pMHO1CAT), and CAT assays were performed after hy-

peroxia exposure. Time-course experiments showed initial rise of CAT activity at 8 h of hyperoxia, with peak CAT activity after 24 and 48 h of hyperoxia. Figure 8 shows the mean fold induction of CAT activity in stably transfected pMHO1CAT cells after 24 h of hyperoxia compared with control, untreated pMHO1CAT cells. We then stably transfected RAW 264.7 cells with the HO-1 extended promoter linked to a CAT reporter gene (pMHO1CAT), and CAT assays were performed after hyperoxia exposure. We also stably transfected RAW 264.7 cells with pMHO3CAT, a 5' flanking region of the HO-1 gene up to but not containing the SX2 enhancer fragment, linked to the CAT reporter gene, and CAT assays were performed after hyperoxia. We did not observe any increase of CAT activity in either pMHO1CAT- or pMHO3CAT-transfected cells after hyperoxia (Figure 8). In

TABLE 2  
Effect of actinomycin D and cycloheximide on hyperoxia-induced HO-1 mRNA expression

Treatment	Relative mRNA level
Control	1.0
Actinomycin D	< 1.0
Cycloheximide	6.3
95% oxygen	13.7
95% oxygen + actinomycin D	< 1.0
95% oxygen + cycloheximide	11.3

Cells were pretreated with actinomycin D (0.1  $\mu$ M/ml) or cycloheximide (1.0  $\mu$ M/ml) for 1 h before hyperoxia exposure. Actinomycin D and cycloheximide were left in cultures during hyperoxia exposure. RNA was harvested 24 h after hyperoxia and analyzed for HO-1 mRNA expression by Northern blot analysis. Data are expressed as relative HO-1 mRNA levels compared with untreated control samples, and are mean values from three independent experiments.



**Figure 7.** Kinetics of HO-1 protein expression in RAW 264.7 cells after hyperoxia. Total protein was isolated from cells after hyperoxia exposure or endotoxin (1  $\mu$ M/ml) and subjected to Western blot analysis. 1: Normoxia control; 2: 8 h endotoxin; 3: 24 h endotoxin; 4: 8 h hyperoxia; 5: 24 h hyperoxia. Data shown is a representative blot of three independent experiments.

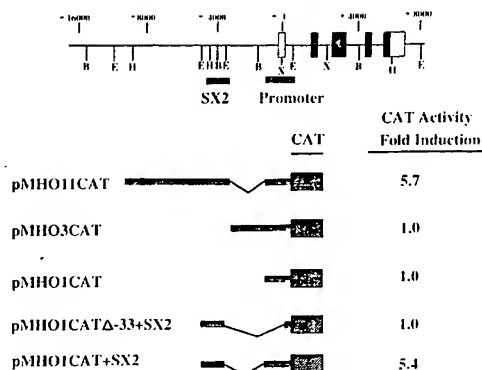


Figure 8. Deletion analysis of the HO-1 gene in RAW 264.7 cells after hyperoxia. Stably transfected RAW 264.7 cells of indicated CAT constructs were exposed to hyperoxia for 24 h and then analyzed for CAT activity. Data represent the mean fold induction of CAT activity in cells exposed to hyperoxia compared with cells in normoxia from three or four independent experiments. A partial restriction map and structural organization of the mouse HO-1 gene is shown with exons marked by open boxes (untranslated regions) and solid boxes (protein coding regions). Position +1 denotes the transcription initiation site. The location of the enhancer fragments SX2 and promoter are indicated by solid black bar. B, Bam HI; E, EcoRI; H, HindIII; X, XhoI.

view of these observations that pMHO1CAT and pMHO3CAT were not sufficient for HO-1 gene transcription, whereas pMHO1CAT was effective in inducing transcriptional activity of HO-1 after hyperoxia, we then hypothesized that one mechanism of pMHO1CAT activation by hyperoxia may be mediated by a 5' distal enhancer fragment (SX2) located 4 kb from the transcription site of the HO-1 gene. We have recently observed that the SX2 distal enhancer fragment of the HO-1 gene alone can mediate endotoxin-induced HO-1 gene transcription (22). Thus, RAW 264.7 cells were stably transfected with a plasmid containing the SX2 enhancer fragment linked to the minimal promoter and the CAT reporter gene (pMHO1CATΔ-33+X2), and CAT assays were performed after hyperoxia exposure. Surprisingly, we did not observe any increase in CAT activity in these cells after hyperoxia. However, when we stably transfected SX2 distal enhancer fragment of the HO-1 gene and the HO-1 extended promoter linked to the CAT gene (pMHO1CAT+X2) (Figure 8) into RAW 264.7 cells and analyzed for CAT activity after hyperoxia, we observed increased CAT activity of these stably transfected pMHO1CAT+X2 cells at 8 h of hyperoxia with peak activity at 24 and 48 h of hyperoxia. Figure 8 shows the mean fold induction of CAT activity of pMHO1CAT+X2 cells after 24 h of hyperoxia compared with untreated pMHO1CAT+X2 cells.

We also examined whether posttranscriptional regulation plays an important role in increased HO-1 gene expression after hyperoxia. Pretreatment of RAW 264.7 cells with the protein synthesis inhibitor cycloheximide had negligible effect on hyperoxia-induced HO-1 gene expression, suggest-

ing that *de novo* protein synthesis is not required for increased HO-1 gene expression after hyperoxia (Table 2). Interestingly, cycloheximide alone resulted in a modest induction of HO-1 mRNA. For cycloheximide experiments, cells were treated with polymyxin B (10 μg/ml) to control for possible endotoxin contamination.

#### Effect of Hyperoxia on HO-1 mRNA Stability

Since hyperoxia could also increase steady-state HO-1 mRNA levels by enhancing the stability of HO-1 mRNA transcripts in addition to transcriptional activation, we determined whether hyperoxia affected the mRNA turnover of HO-1 mRNA. Alterations in mRNA turnover were assessed by pretreating cells with hyperoxia followed by the addition of the RNA synthesis inhibitor actinomycin D in the presence or absence of hyperoxia exposure. Total RNA was isolated at the indicated times for Northern blot analyses, and the data shown in Figure 9 are represented as percent mRNA remaining, relative to that present at time 0. Figure 9 shows that hyperoxia exerted negligible effect on HO-1 mRNA stability.

#### Transcription Factor AP-1 Is Activated *in vitro* after Hyperoxia Exposure

Since the HO-1 gene promoter and the SX2 5' distal enhancer fragment contain putative AP-1 binding sites, we reasoned that AP-1 motifs may be functionally active and may mediate HO-1 gene induction in cells after hyperoxia exposure. Electrophoretic mobility shift assays using a synthetic, double-stranded DNA probe specific for the consensus AP-1 binding site demonstrated increased AP-1 binding activity in RAW 264.7 cells after 1, 4, and 24 h of hyperoxia, with peak bind-

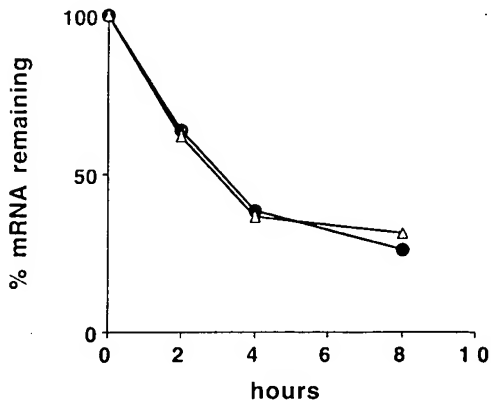


Figure 9. Effect of hyperoxia on mRNA stability of HO-1 in RAW 264.7 cells after hyperoxia. Cells were exposed to hyperoxia for 24 h, after which point actinomycin D (0.1 μg/ml) was added to the cells (time 0). Total RNA was then extracted at the indicated times and analyzed for HO-1 expression by Northern blot analysis. (Δ) Hyperoxia + actinomycin D; (●) normoxia + actinomycin D. Each data point represents the average value from five independent experiments.

ing activity after 4 h (Figure 10A) of hyperoxia exposure compared with normoxic control cells. The specificity of AP-1 binding activity was demonstrated by the ability of an unlabeled AP-1 oligonucleotide to compete with the radiolabeled AP-1 sequence for binding of nuclear factors (Figure 10A), whereas an unlabeled Sp1 oligonucleotide containing an unrelated consensus sequence did not compete with the radiolabeled AP-1 probe (Figure 10B). To delineate the specific AP-1 proteins responsible for DNA binding activity in cell lysates following hyperoxia, gel mobility supershift assays were performed using antibodies for either c-Fos or c-Jun. As shown in Figure 10B, incubation of the DNA-protein complex with antibodies to both c-Fos and c-Jun resulted in new supershifted retarded bands (*arrows*). Addition of a nonspecific antibody had no effect on the AP-1 complex, with absence of supershifted band. The lack of a completely supershifted band with Fos/Jun antibodies may reflect the inability of the antipeptide antibodies to recognize complexed proteins as observed in other cell systems (39–41).

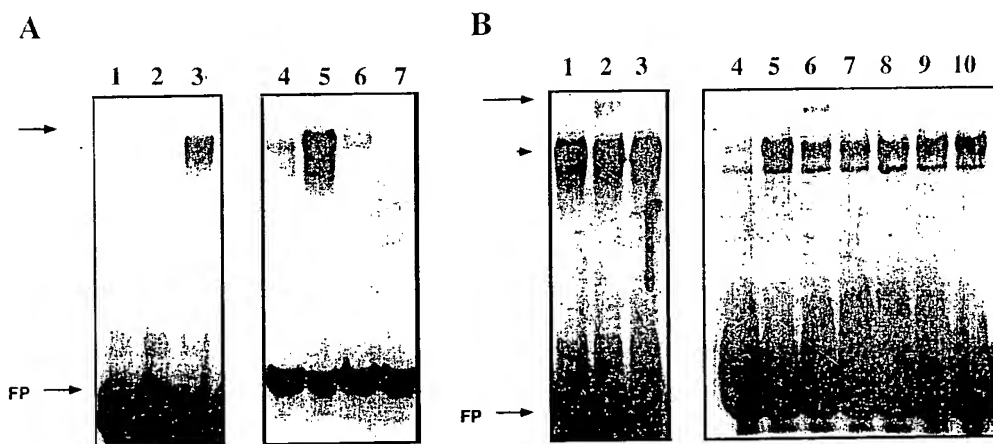
### Discussion

Hyperoxia has been widely used to study the pathogenesis of oxidant lung injury. Damaging effects of hyperoxia are mediated by ROS including superoxide and hydroxyl radicals, and hydrogen peroxide. The cellular and molecular responses of the lung to the deleterious effects of these ROS in hyperoxic injury involve alterations in gene expression of AOE and stress-response gene products (9, 13). In this study

we show that expression of HO-1, a 32-kD stress-inducible gene, is also highly stimulated in the lung *in vivo* in response to hyperoxia. Interestingly, the regulation of HO-1 expression *in vivo* is quite different from those observed for other AOE. We show that the increased HO-1 mRNA levels after hyperoxia correlated with similar increases in HO-1 protein and enzyme activity. In contrast, studies by Clerch and Masaro have shown increased mRNA levels of MnSOD by hyperoxia, whereas mRNAs for CuZnSOD, catalase, and glutathione peroxidase do not change. Additionally, the enzyme activity levels of these AOE are variable, since hyperoxia increases catalase and glutathione peroxidase activities and decreases MnSOD enzyme activity, but does not alter CuZnSOD enzyme activity (9).

Interestingly, hyperoxia also increased HO-1 mRNA levels in the various systemic rat organs. This may reflect the systemic effects of inflammatory cytokines such as interleukin-6 (IL-6), IL-1, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which all are potent inducers of HO-1 expression *in vivo* (42, 43) and have been shown to be induced by hyperoxia *in vivo* (44). It is also plausible that the increased HO-1 expression in the various tissues resulted from direct effects of hyperoxia, since we have previously shown that high O<sub>2</sub> tensions (> 500 mm Hg) can be achieved in arterial blood in the rat with up to 56 h of hyperoxia exposure, and that O<sub>2</sub> tensions correlated with expression of AOE genes (13).

Localization of HO-1 expression by immunohistochemical studies of the rat lung after hyperoxia was similar to the



**Figure 10.** Electrophoretic mobility gel shift of cellular protein extracts for AP-1 binding activity after hyperoxia exposure. (A) Nuclear protein extracts were obtained from RAW 264.7 cells after hyperoxia and analyzed for AP-1 binding activity using a 22-mer double stranded oligonucleotide probe containing an AP-1 site. 1: Free probe; 2: normoxia control; 3: 4 h hyperoxia; 4: normoxia control; 5: 4 h hyperoxia; 6: 4 h hyperoxia and cold 10  $\times$  AP-1; 7: 4 h hyperoxia and cold 10  $\times$  AP-1. FP, free probe. Arrow = AP-1 complex. Arrowhead = supershifted band. Data shown are representative gel of five independent experiments. (B) Gel mobility supershift of AP-1 complex in RAW 264.7 cells after hyperoxia. Equal molar concentrations of c-Fos or c-Jun antibody were added to nuclear protein extracts after incubation with labeled AP-1 probe. 1: 4-h hyperoxia; 2: 4-h hyperoxia with antibody to c-Fos; 3: 4-h hyperoxia with antibody to c-Jun; 4: normoxia control; 5: 4-h hyperoxia; 6: 4-h hyperoxia with antibody to c-Fos; 7: 4-h hyperoxia with non-specific IgG antibody; 8: 4-h hyperoxia and 1  $\times$  cold Sp1; 9: 4-h hyperoxia and 10  $\times$  Sp1; 10: 4-h hyperoxia and 100  $\times$  Sp1. FP, free probe; Arrowhead = AP-1 complex; Arrow = supershifted band. Data shown are representative gel of three independent experiments.

pattern observed in endotoxin-induced HO-1 expression (22). The bronchoalveolar epithelium and inflammatory cells were responsible for much of the increased HO-1 staining in the rat lung after hyperoxia. We also observed increased HO-1 mRNA expression in a variety of cultured cells, both cell lines and primary culture cells, after hyperoxia exposure *in vitro*. This induction of HO-1 mRNA in cultured cells, in the absence of an *in vivo* milieu and inflammatory response, suggests that leukocyte-derived ROS are not an absolute requirement for increased HO-1 gene expression, and that hyperoxia directly or indirectly can transduce intracellular signals to induce HO-1 gene expression. Pretreatment of cells with cycloheximide failed to block HO-1 gene expression after hyperoxia, suggesting that new protein synthesis is not required for HO-1 induction. Cycloheximide has also been shown not to block IL-6- and endotoxin-induced expression of HO-1 in human hep3B cells and RAW 264.7 macrophages, respectively (22, 45). Cycloheximide also failed to block endotoxin-induced expression of MnSOD (46).

Much interest has been generated by investigators to understand the molecular regulation of HO-1 expression, not only because induction of the HO-1 gene by heme and non-heme inducers is controlled almost entirely at the level of gene transcription (21, 22, 26, 34-36), but also because it is clear from recent evidence that HO-1 induction plays a critical role in providing protection against oxidant insult *in vivo* and *in vitro*. Very little if any information exists regarding the molecular regulation of HO-1 expression after hyperoxic oxidant stress. Thus, to further delineate the molecular regulation of HO-1 gene induction after hyperoxia, we chose to use macrophage cells for the remainder of our studies, for the following reasons. First, the level of HO-1 mRNA expression was most abundant in the inflammatory cells *in vivo* after hyperoxic exposure, based on our immunohistochemical studies (Figure 4). Second, the kinetics and level of HO-1 mRNA induction after hyperoxia exposure (Table 1; and Figure 6B) were most dramatic in the macrophages. Third, very little is known regarding the effects of hyperoxia on macrophage gene expression. Similar to our observations *in vivo*, increased HO-1 mRNA levels were accompanied with similar increases in HO-1 protein in RAW 264.7 macrophages. In contrast, the regulation of other AOE expression *in vitro* is quite different, since hyperoxia induces increased mRNA expression for AOE such as MnSOD, CuZnSOD, glutathione peroxidase, and catalase, but decreases catalase activity, increases CuZnSOD and glutathione peroxidase activities, and does not alter MnSOD activity in endothelial cells (47).

We then proceeded to show that HO-1 induction by hyperoxia is dependent on gene transcription. This transcription-dependent expression of the HO-1 gene has also been observed following other stimuli, including hydrogen peroxide, ultraviolet irradiation, heme, cadmium, endotoxin and sodium arsenite (22, 26, 34-36). Surprisingly, the distal enhancer (SX2) alone, with the minimal promoter (PMHOICATΔ-33+ SX2), was not sufficient to induce HO-1 gene transcription after hyperoxia (Figure 8), whereas our previous observations have shown that these PMHOICATΔ-33+ SX2 cells were greatly induced by stimuli such as endo-

toxin and cadmium (22, 35), suggesting a different upstream pathway of HO-1 activation between these stimuli. It is intriguing, however, that when the distal enhancer (SX2) is linked to the extended promoter (PMHOICAT+ SX2), induction of HO-1 gene transcription was observed, whereas the extended promoter alone (pMHOICAT) had no effect on HO-1 gene transcription after hyperoxia. The majority, if not all, of increased HO-1 gene transcription after hyperoxia was mediated by both the extended HO-1 promoter and the 5' distal enhancer (SX2), in that the relative fold induction of CAT activity of PMHOICAT cells were quite similar to those observed in the PMHOICAT+ SX2 cells after hyperoxia (Figure 8). Taken together, these observations strongly implicate an important cooperation between transcription factors bound to elements within the extended promoter and the 5' distal enhancer (SX2) for HO-1 gene transcription after hyperoxia. Increasing evidence has appeared in the literature showing cooperation between various DNA binding proteins such as the AP-1 complex and C/EBP proteins, and the AP-1 complex and nuclear factor-κB (48, 49).

Although it is beyond the scope of this study to clearly identify the cognate DNA-binding proteins in the promoter and enhancer elements that cooperate to activate HO-1 gene transcription after hyperoxia, it is quite plausible that AP-1 transcription factors represent suitable candidates to participate in this cooperation, for the following reasons. First, we have evidence of AP-1 activation in these cells after hyperoxia by gel shift assays and supershift of the AP-1 complex with c-Fos and c-Jun. Second, there are two consensus AP-1 binding sites in the 5' distal enhancer fragment (SX2) (34, 35) and putative AP-1 binding elements in the promoter. Third, we have already shown previously by deletion and mutational analyses that AP-1 elements within the SX2 enhancer mediate HO-1 gene transcription after inducers such as endotoxin, phorbol esters, and cadmium (22, 34). Fourth, many studies have demonstrated transcriptional activators, including AP-1, to play important roles in mediating the early events in molecular responses to oxidative stress (50, 51), and AP-1 binding sites have been identified in AOE genes such as MnSOD (52). It is interesting to note that the antioxidant-response element (ARE) consensus sequence (TGACNNNGC) bears homology with the AP-1 core sequence (TGA C/G T C/A A). The ARE has been shown to mediate the induction of genes such as the rat NAD (P)H:quinone reductase and glutathione S-transferase Ya subunit genes by xenobiotics and electrophiles (53, 54). Although we cannot absolutely rule out the possibility that the ARE may also play a role in our studies, we feel that AP-1 is still a more likely candidate based on the reasons discussed above. In addition, not surprisingly, the electrophiles are the only inducers we have observed to involve the ARE in the activation of the HO-1 gene (54).

In conclusion, our study shows marked *in vivo* and *in vitro* HO-1 induction after hyperoxia. HO-1 gene activation is transcriptionally regulated after hyperoxia and very interestingly is dependent on cooperativity between the extended promoter and the 5' distal enhancer fragment SX2 of the HO-1 gene. Current and future studies are directed in identifying the precise DNA binding elements of the promoter and the distal enhancer that are involved in the transcrip-

tional regulation of HO-1 gene by hyperoxia. Additionally, we are examining the functional significance of HO-1 induction in hyperoxic lung injury by using transgene approach to assess whether increased production of HO-1 *in vivo* selectively in the lung provides protection against hyperoxia.

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